# Unraveling the stepwise activation mechanism of HacA, the key regulator of the unfolded protein response in Aspergillus niger

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# Chapter 2

# The Transcription Factor HacA Mediates the Unfolded Protein Response in *Aspergillus niger*, and Up-Regulates its Own Transcription

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# Abstract

The unfolded protein response (UPR) involves a complex signalling pathway in which the transcription factor HacA plays a central role. Here we report the cloning and characterisation of the *hacA* gene and its product from *Aspergillus niger*. Endoplasmic reticulum (ER) stress results in the splicing of an unconventional 20 nt intron from the *A. niger hacA* mRNA, and in addition to the truncation of the 5'-end of the *hacA* mRNA by 230 nt. In this study the UPR was triggered by over-expressing tissue plasminogen activator (t-PA), and by treatment of mycelia with dithiothreitol (DTT) or tunicamycin. Over-expression of the processed form of *hacA* not only led to the upregulation of *bipA*, *cypB* and *pdiA*, mimicking an UPR, but also led to the upregulation of the *hacA* gene itself. *In vitro* binding assays confirmed that the HacA protein binds to the promoters of genes encoding ER-localised chaperones and foldases, and to the promoter of *hacA* gene itself. Finally, a GFP-HacA fusion was shown to localise in the nucleus.

# Introduction

The endoplasmic reticulum (ER) serves as a folding compartment for membrane and secretory proteins. In the oxidising environment of the ER, protein folding is assisted by numerous protein chaperones and foldases [39], and a variety of quality control mechanisms ensure that only correctly folded proteins are released [30,190]. A variety of physiological or environmental stress conditions can disturb protein folding and lead to the accumulation of unfolded proteins in the ER. Cells respond to such an accumulation by increasing the transcription of genes that code for ER-resident chaperones and foldases, thereby increasing the folding capacity of the compartment. The signalling pathway involved is part of the unfolded protein response (UPR) [64,123,150,179]. Genome-wide analysis in yeast, using DNA micro arrays, has linked a large number of genes to the UPR, with functions far beyond those ER chaperones [38,160]. By affecting virtually every stage of the secretory pathway, the UPR appears to be a versatile regulatory system that serves to maintain the homeostasis of ER functions under stress conditions. The key step in the activation of the UPR in yeast is the splicing of an unconventional intron from *HAC1* mRNA, which serves to relieve a translational block. [66,137]. *HAC1* mRNA encodes the basic leucine zipper (bZIP)-type transcription factor Hac1p, which directly activates the transcription of UPR genes. Splicing of the yeast *HAC1* mRNA occurs in a non-spliceosomal manner. During ER stress, the intron borders of *HAC1* mRNA are cleaved by Ire1p [43,152], after which the exons are rejoined by the tRNA ligase Rlg1p [151]. Ire1p, a kinase/RNase which resides in the ER

membrane senses the accumulation of unfolded protein via a dynamic interaction with the chaperone BiP [8]. Release of BiP triggers the dimerisation and subsequent trans-phosphorylation of Ire1p, thereby activating the UPR. In animal cells, nearly all the features of the yeast UPR splicing system are conserved, but in comparison with yeast their ER stress pathway appears to be more extensive [148,186]. The transcription factor XBP1, which is involved in the ER stress response of *Caenorhabditis elegans* and mammalian cells, is activated through intron splicing mediated by IRE1, in a manner similar to that seen in yeast. In addition, transcription of the *XBP1* gene is induced by ATF6, an ER membrane associated transcription factor, which is proteolytically released during ER stress [81].

In this study we focus on the unfolded protein response in the filamentous fungus *Aspergillus niger*, an industrial production organism which is capable of secreting large amounts of native proteins like glucoamylase. Yields of heterologous proteins, however, are often low [2]. In order to improve *A. niger* as a host for homologous and heterologous protein production, a detailed knowledge about the functioning of the secretion pathway and its bottlenecks is important. Here we report the isolation and characterisation the *A. niger hacA* gene, the functional homologue of the yeast *HAC1*, which codes for the transcriptional activator of the UPR.

## Materials and Methods

### Strains, Culture Conditions and Transformations

*Escherichia coli* strains TOP10 and DH5α (Invitrogen) were used as hosts to propagate plasmids. The cDNA library from *Aspergillus niger* var. *awamori* strain UVK143f was constructed in the vector pYES2 (Invitrogen). *A. niger* N592 (*cspA1, pyrA5*) was used as the recipient strain for transformation, and *A. niger* N402 (*cspA1*), and *A. niger* D15::p*glaA*-t-PA#19, which produces the human tissue plasminogen activator protein (t-PA) [181], were used to investigate the UPR under different stress conditions.

All *A. niger* strains were grown on minimal media (MM) plates containing per litre 6 g $NaNO_3$, 1.5 g $KH_2PO_4$, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.5 g KCl, trace elements as described by Vishniac and Santer [172] and 20 g fructose as a carbon source (pH 6.0). Liquid cultures were grown in MM supplemented with 2 g casamino acids and 5 g yeast extract (CM), and inoculated with $10^6$ spores/ml and incubated at 30°C. All growth experiments for subsequent Northern analysis were started in fresh CM media with 1.5 g mycelia of an overnight culture. Except for *A. niger* D15::p*glaA*-t-PA#19, which was transferred to MM with 20 g maltodextrin, for induction of the glucoamylase promoter. ER stress was provoked by adding either 3 mg/ml dithiothreitol (DTT) or 10 µg/ml tunicamycin. All $t_0$ samples were taken just before the transfer to the experimental medium.

*A. niger* protoplasts were co-transformed according to the procedure of Kusters–van Someren *et al.* [76] with (1) plasmid pHM50, which expresses the coding region of *hacA* without the 20 nt unconventional intron (*hacA*$^{i}$) under the control of the constitutive *gpdA* promoter and the *trpC* terminator, and (2) pGW635, which contains the *pyrA* gene of *A. niger* [45]. pHM52Δ4, which expresses a *gfp-hacA*$^{i}$ fusion under control of the *gpdA* promoter, also contains the *pyrA* gene, thus allowing direct transformation of *A. niger* N592.

**Gene Isolation**

RNA was extracted from *A. niger* var. *awamori* strain UVK143f after growth of mycelium on glucose, starch or beet pulp as a sole carbon source, and from mycelium grown under nitrogen starvation. The RNAs were mixed and used for library construction [174]. Southern hybridisation and screening of the cDNA library by colony hybridisation were performed as described by Sambrook *et al.* [141]. The library colony hybridisation was done overnight in a hybridisation mix without formamide [141] at 58°C. Subsequently the filters were washed in 2xSSC, 0.1% SDS for 2x 5 minutes at room temperature, and with the same solution at 58°C for 30 minutes. PCR based genomic walking was performed as described by Siebert *et al.* [153] to isolate the flanking regions of the *hacA* gene. DNA sequencing reactions were performed with the Bigdye sequencing kit (Applied Biosystems) and analysed with the ABI Prism 3100 Genetic Analyser (Applied Biosystems).

**Tabel I:** Oligonucleotide primers used in this study

| Primer | Sequence | Site |
|---|---|---|
| P1 | 5'-ACCATGGTGGAAGAAGCATTCTCTCCAG | *NcoI* |
| P2 | 5'-CCGCTGCAGGATGTTGTGTCA | *PstI* |
| P3 | 5'-GACCTGCAGTGTCCGTCGCTG | *PstI* |
| P4 | 5'-GGGATCCTAACAGCCAGCTGCAATGCCCTG | *BamHI* |
| P5 | 5'-GGGATCCTAGTGGTGGTGGTGGTGGTGACAG CCAGCTGCAATGCCCTG | *BamHI* |
| P6 | 5'-GGGCGCCTCCATGATGGAAGAAGCATTCTCTC | *NarI* |
| P7 | 5'-GCCATGGTGAGCAAGGGCGAGGAG | *NcoI* |
| P8 | 5'-CGGCGCCGGACTTGTACAGCTCGTCCATGCC | *NarI* |
| P9 | 5'-CGTCGAGAACGTCAAAGGCGAACCCGTC | |

**Plasmid Construction**

For over-expression of *hacA* without the need for a stress induced splicing event, and for construction of a *gfp-hacA*$^{i}$ fusion, the *hacA* ORF was amplified in two fragments from *A. niger* cDNA by PCR. Joining of the two gene fragments at the *PstI* site resulted in reconstitution of the *hacA* ORF without its unconventional intron. The primers used in this study are listed in Table 1, and the positions of the primers used to amplify *hacA*$^{i}$ are indicated in Figure 1.

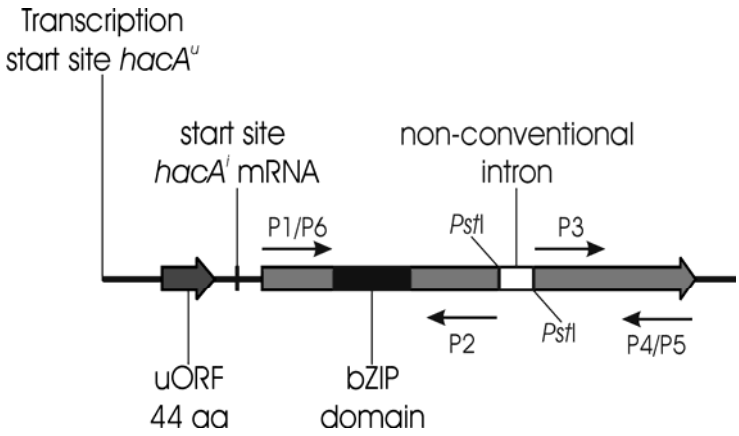


**Figure 1.** Schematic representation of *hacA*$^{u}$ mRNA with its main features. The locations of oligonucleotide primers used for the construction pHM50, pHM52Δ4, and pHM30Δ3 are indicated by the small arrows.

For over-expression in *A. niger* the 5' and 3' portions of the *hacA* ORF were amplified using the primer sets P1-P2 and P3-P4, respectively. The two fragments were then fused at the *PstI* site and cloned into pAN52-1Not. *A. niger* N592 was transformed with the resulting plasmid (pHM50). For expression in *E. coli* a construct was generated in the same way, except that primer P4 was replaced by primer P5, which codes for a $(His)_6$ tag at the C-terminus of the HacA protein. This construct was cloned into pET3d+ (Novagen), and the resulting plasmid (pHM30Δ3) was transformed into the *E. coli* strain BL21 (DE3)pLysS (Novagen). HacA-$(His)_6$ was purified by FPLC (Amersham Pharmacia Biotech), using a Ni-NTA Superflow column (Qiagen), followed by gel filtration on a Superdex 75 column (Amersham Pharmacia Biotech). For construction of a *gfp-hacA*$^{i}$ fusion, *hacA*$^{i}$ was amplified with the primers P4 and P6 using pHM50 as template, and *gfp* was generated with primers P7 and P8 using pEGFP (Clontech) as template. The two genes were ligated together at the *NarI* site, and cloned into pAN52-1Not. Subsequent ligation of the *pyrA* gene into this plasmid resulted in pHM52Δ4, which was used to transform *A. niger* N592. Maps of the plasmids used in this study are shown in Figure 2.

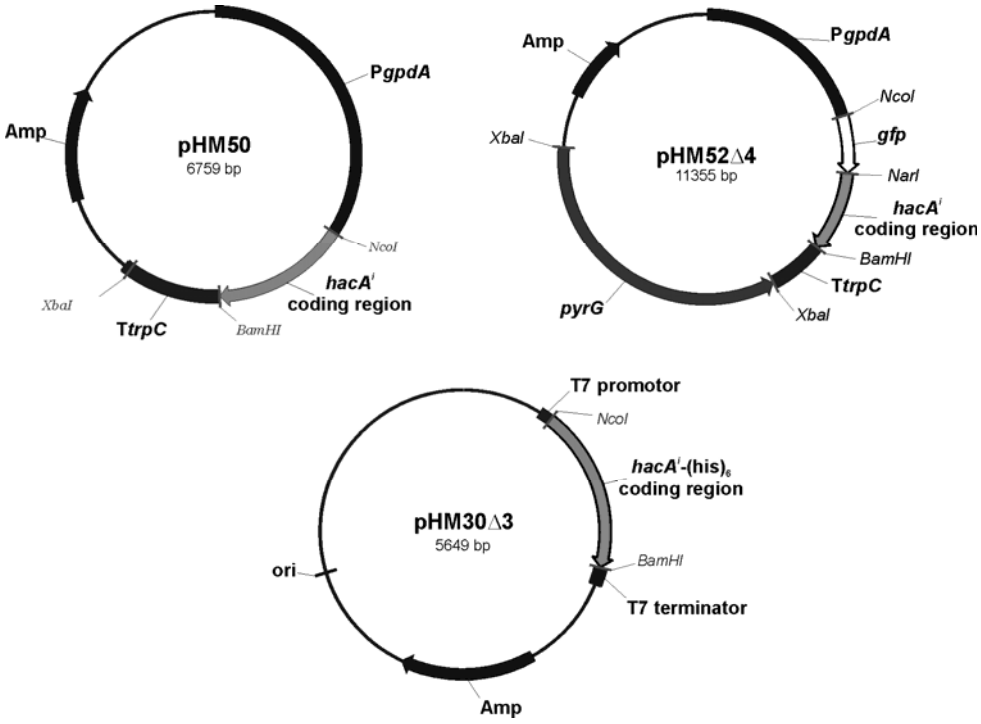


**Figure 2.** Maps of the plasmids used in this study.

## Northern Blot Analysis

For RNA isolations, mycelium was ground with 1mm glass beads using the Fast Prep FP120 (BIO 101 Savant), and total RNA was isolated using the RNeasy plant total RNA kit (Qiagen). For Northern analysis 10 µg aliquots of total RNA were fractionated on a 1.5% (w/v) agarose-formaldehyde gel and blotted onto a Hybond N membrane (Amersham Pharmacia Biotech). To generate probes, DNA fragments were $\alpha$-$^{32}$P-labelled using the Prime-It II labelling kit (Stratagene), and the probes were purified on Biogel P30 spin columns (Biorad). Northern hybridisations were performed at 42°C in 50% formamide containing 10% polyethylene glycol 6000, 5x Denhardt's solution, 50 mM TRIS-HCl (pH 7.5), 1 M NaCl, 0.1% sodium pyrophosphate, 1% SDS and 100 µg/ml salmon sperm DNA (Sigma). Filters were washed 20 minutes with 0.2 x SSC / 0.1% SDS at 60°C.

**Western Blot Analysis**

Criterion SDS-polyacrylamide (PAGE) gels (Biorad) were used for protein electrophoresis. SDS-PAGE gels were stained with Coomassie Brilliant Blue R250 (Biorad) or were blotted onto pure nitrocellulose membranes (Biorad) for Western blot analysis. Protein concentrations were determined according to Bradford [9] using BSA as a standard. Western analysis was performed using standard procedures [141]. Membranes were incubated in 100 ml TN (50 mM TRIS-HCl, 150 mM NaCl, pH 8.0) containing 5 g low fat milk powder and polyclonal antibodies specific for BIPA, CYPB, and PDIA. Immunoreactive proteins were detected using an HRP-conjugated secondary antibody and ECL detection reagents (Amersham Pharmacia Biotech).

**Southern Blot Analysis**

Genomic DNA for Southern analysis was isolated by adding extraction buffer (100 mM TRIS-HCl pH 8.0, 50 mM EDTA, 500 mM NaCl, 1mM DTT, 35 mM SDS) to ground mycelium. After 10-min incubation at 65 °C, mycelium debris was separated by centrifugation, and the DNA in the supernatant was precipitated with isopropanol. The DNA pellet was resuspended in $H_2O$ and incubated with DNase-free RNase (Roche Molecular Biochemicals). For each digest 10 µg of genomic DNA was incubated over night at 37 °C with either 20 U of either *NotI* or *NcoI* endonuclease. The reactions were fractionated on a 0.8% TAE agarose gel. Blotting and hybridisation were done according to standard procedures [141].

**5'RACE**

5'RACE (Rapid Amplification of cDNA Ends) was performed on 1 µg of total RNA using the SMART RACE cDNA Amplification Kit (Clontech). Primer P9 (Table 1) was used to amplify *hacA* cDNA.

**Electrophoretic Mobility Shift Assay (EMSA)**

The promoter fragments of *bipA*, *cypB*, *pdiA*, *prpA*, and *tigA* (Table 2) were synthesised by PCR performed on gDNA of *A. niger*. The resulting fragments were gel purified and end-labelled with $\gamma$-$^{32}$ATP using T4 polynucleotide kinase (New England Biolabs) and purified on Biogel P30 spin columns (BioRad). Binding reactions (40 µl) included 0.5 µg of purified HacA protein, and 1x binding buffer (20 mM HEPES pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.25 mM EDTA, 0.5 mM DTT, 2% Ficoll 200, 100 µg/ml poly(dI-dC), and 5% glycerol). 1 ng of radiolabelled probe was added and binding was allowed to proceed on ice for 15 min. The reactions were loaded on a 5% polyacrylamide gel and electrophoresed at 100 V for 3 hr in 0.25 x TBE.

**Table 2.** Promoter fragments used in EMSAs

| Gene | **Accesion No.** | **Position relative to ATG** |
|---|---|---|
| *bipA* | Y08868 | -412 to 0 |
| *cypB* | AY005867 | -396 to 0 |
| *pdiA* | X98797 | -432 to 0 |
| *prpA* | AF095899 | -587 to 0 |
| *tigA* | X98748 | -330 to 0 |
| *hacA* | AY303684 | -863 to -265 |

### UV Microscopy

Transformants expressing GFP-HacA were grown in minimal medium as described above. Mycelium samples were examined by fluorescent microscopy on a Polyvar microscope (Reichert-Jung) coupled to a cooled CCD digital camera, using FITC filters. Pictures were analysed with the Metamorph software package (version 4.5). DAPI (Molecular Probes) was used at a concentration of 1 µM for visualisation of nuclei.

## Results

### Cloning and Sequence Analysis

The gene encoding the transcriptional activator of the unfolded protein response in *A. nidulans*, *hacA* , has previously been cloned based on its homology to the *HAC1* gene of *S. cerevisiae* [140]. The *A. nidulans hacA* sequence was used in this study as a probe to facilitate cloning of the corresponding gene from a cDNA library derived from the *A. niger* var. *awamori* strain UVK143f. Several positive clones were obtained, and sequencing of their 5' ends revealed that they were indeed derived from a homologue of the *A. nidulans hacA* gene. Based on the DNA sequence, primers were designed, and a 6.2-kb genomic fragment including the *hacA* coding sequence together with its flanking regions (Accession No. AY303684) was cloned from *A. niger* N402 by genomic walking. Sequence comparison of the *hacA* cDNA from *A. niger* var. *awamori* with the *hacA* gene from *A. niger* N402 revealed that the two encode identical proteins.

In *S. cerevisiae*, an unconventional 252-bp intron is spliced from the *HAC1* mRNA during UPR, whereas a much shorter (26-bp) intron is excised from the mammalian *XBP1* mRNA under similar conditions [12,22]. The *hac1*/*hacA* genes from *T. reesei* and *A. nidulans* have also been shown to contain non-conventional introns that are spliced during the induction of UPR [140]. Comparison of the cDNA sequence of one of the clones obtained from the cDNA library of *A. niger* var. *awamori* strain UVK143f with the corresponding genomic

sequence revealed a non-conventional intron of 20 nt. The splicing site of the intron could not be determined unambiguously by comparing the unspliced and spliced sequences, because of the presence of a CTGCAG sequence on both sides of the intron. However, the intron borders and splice-sites of the yeast *HAC1* and mammalian *XBP1* are well conserved [12], and these conserved bases were also found at the borders of the *A. niger hacA* (Fig. 3). Moreover, a strong secondary RNA structure was predicted for the intron region and its flanking sequences. The structure of the conserved intron borders—organised in two loops of 7 nt each—was similar to those formed by both the yeast *HAC1* and mammalian *XBP1* introns. Most probably, the *A. niger hacA* transcript is also spliced within these loops.

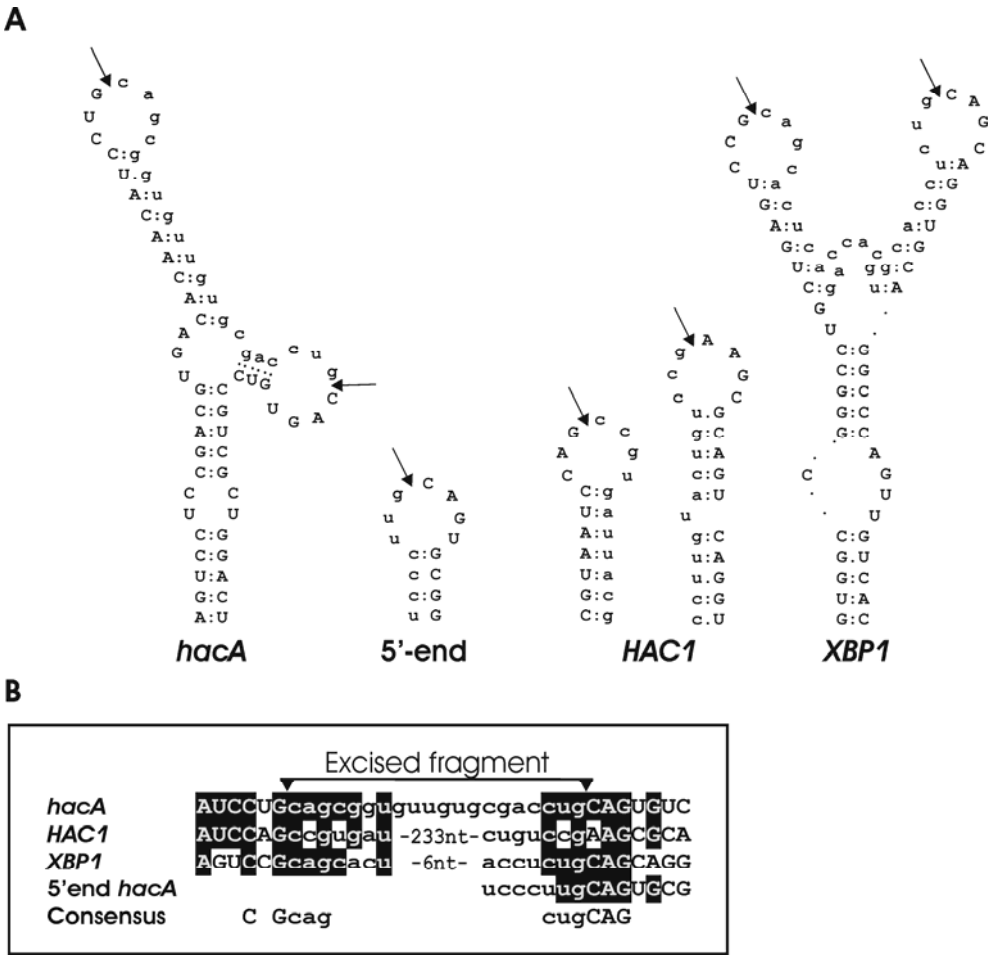


**Figure 3.** Structure and context of the *hacA* unconventional intron. **(A)** Predicted secondary structure of the *A. niger hacA* intron, the 5'-end of the *hacA*$^{i}$ mRNA, the 5' and 3' border of the *S. cerevisiae HAC1* intron, and the mammalian *XBP1* intron, respectively at the RNA level. The intron sequences are shown in lower case letters, and the arrows indicate the cleavage sites. **(B)** Alignment of the mRNA sequence surrounding the unconventional intron in *A. niger hacA*, *S. cerevisiae HAC1*, and the mammalian *XBP1*. The 5'-end of *A. niger hacA*$^{i}$ mRNA is also indicated. Conserved residues in the loops are indicated in the consensus sequence.

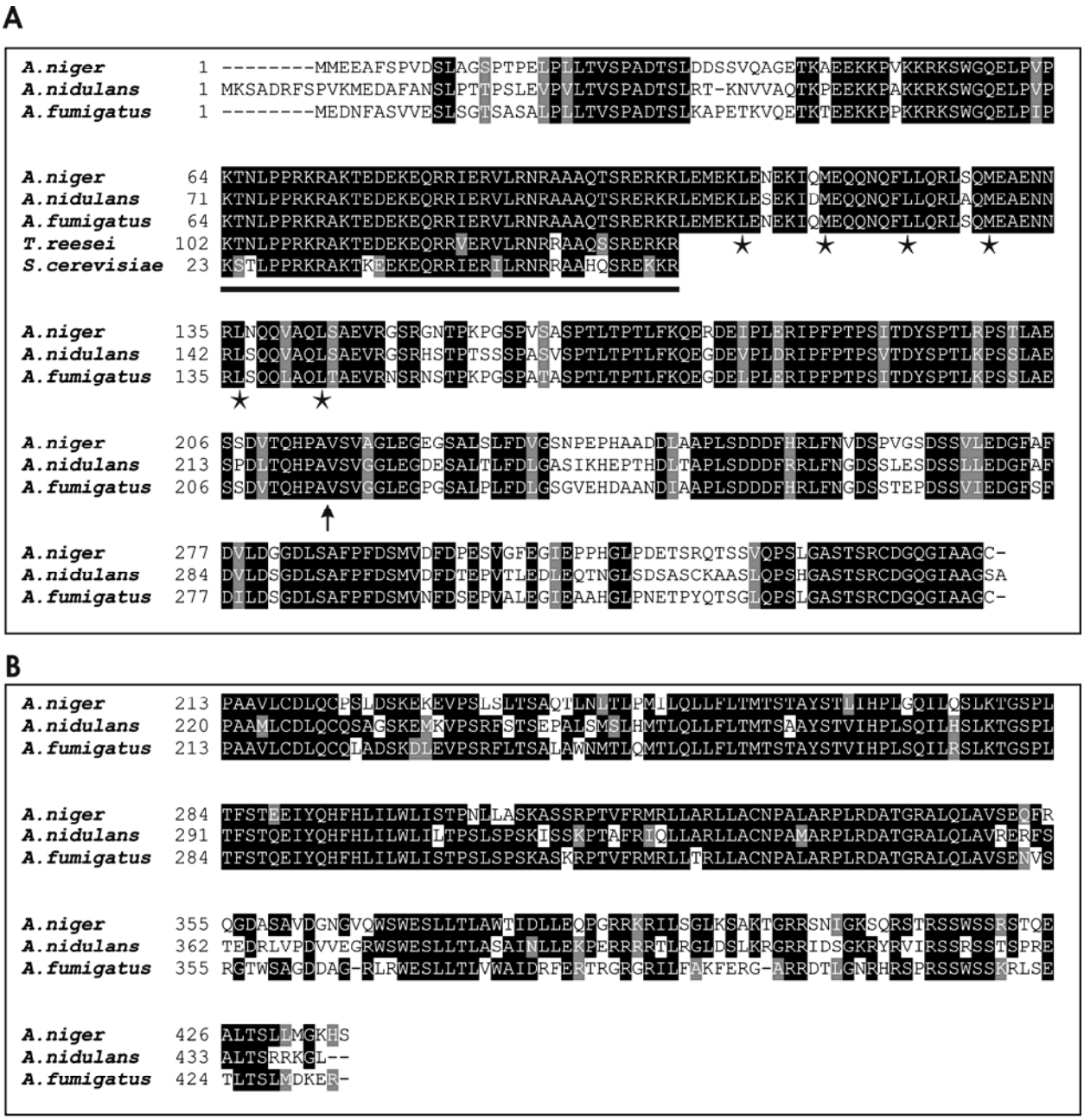


**Figure 4.** Alignment of the amino acid sequences of the induced and uninduced forms of HacA from *A. niger*, *A. nidulans* and *A. fumigatus*. Only the DNA binding domains of the *S. cerevisiae* Hac1p and the *T. reesei* Hac1 proteins are shown. **(A)** The putative DNA binding domain is underlined the residues of the putative leucine zipper are indicated by the *asterisks*, and the position of the unconventional intron in the three *Aspergillus* species is indicated by the arrow. **(B)** Alignment of the uninduced forms of HacA from *A. niger*, *A. nidulans* and *A. fumigatus* from the site of the unconventional intron to the C-terminus.

The unspliced *hacA*$^{u}$ mRNA contains an ORF coding for a 436-amino acid protein. Splicing replaces a C-terminal portion coding for 222 amino acids with a different sequence coding for 128 amino acids. This differs from the situation in yeast, where a 10-residue C-terminal tail is replaced by a sequence of 18 amino acids as a result of splicing [22].

The ORF of the *A. niger hacA*$^{i}$ mRNA encodes a protein of 342 amino acids, which is 73% and 80% identical to its orthologues from *A. nidulans* and *A. fumigatus*, respectively (Fig. 4a). HacA belongs to the bZIP type family of transcription factors, which typically have a leucine-zipper dimerisation motif downstream of the DNA-binding domain. In *A. niger* HacA there are six amino acids (L, M, L, M, L and L) that could potentially form the leucine zipper.

Interestingly, alignment of the protein sequences encoded by the unspliced *hacA*$^{u}$ mRNAs from *A. niger*, *A. nidulans* and *A. fumigatus* showed a high degree of identity (71%–75%) among the species, even in the part downstream of the non-conventional intron (Fig. 4b). This could indicate that the HacA protein encoded by *hacA*$^{u}$ mRNA also has a function for which the C-terminal segment is important.

**Northern Analysis of ER Stress**

One of the key steps in activation of the UPR pathway in yeast and mammals is the Ire1p/IRE1 mediated splicing of a unconventional intron from the mRNA encoding the transcriptional activator of the UPR [12,22]. A similar event has been shown to occur during the induction of the UPR in *T. reesei* and *A. nidulans* [140]. To study this event in *A. niger*, ER stress was triggered by three means: (1) over-expression of a heterologous protein (t-PA), (2) treatment of mycelium with DTT, and (3) treatment of mycelium with tunicamycin. DTT and tunicamycin are both known to be chemical inducers of the UPR. Tunicamycin provokes the UPR by inhibiting the core oligosaccharide addition to nascent polypeptides in the ER, thereby blocking proper folding and transit through the ER [122]. As a reducing agent, DTT disrupts the oxidising environment of the ER, preventing the formation of disulphide bonds. Tissue plasminogen activator (t-PA) is a serine protease which, when activated by fibrin, itself activates plasminogen, enabling the dissolution of blood clots. Low expression levels have hampered efforts to produce useful amounts of t-PA in various host organisms, and in *A. niger* it has been used as a model protein to assess the problems of heterologous protein expression [181]. Since expression of t-PA in *A. niger* leads to the transcriptional upregulation of genes encoding the ER chaperones and foldases BIPA, CYPB, and PDIA, it was chosen as a model strain to assess the UPR under conditions of heterologous protein production.

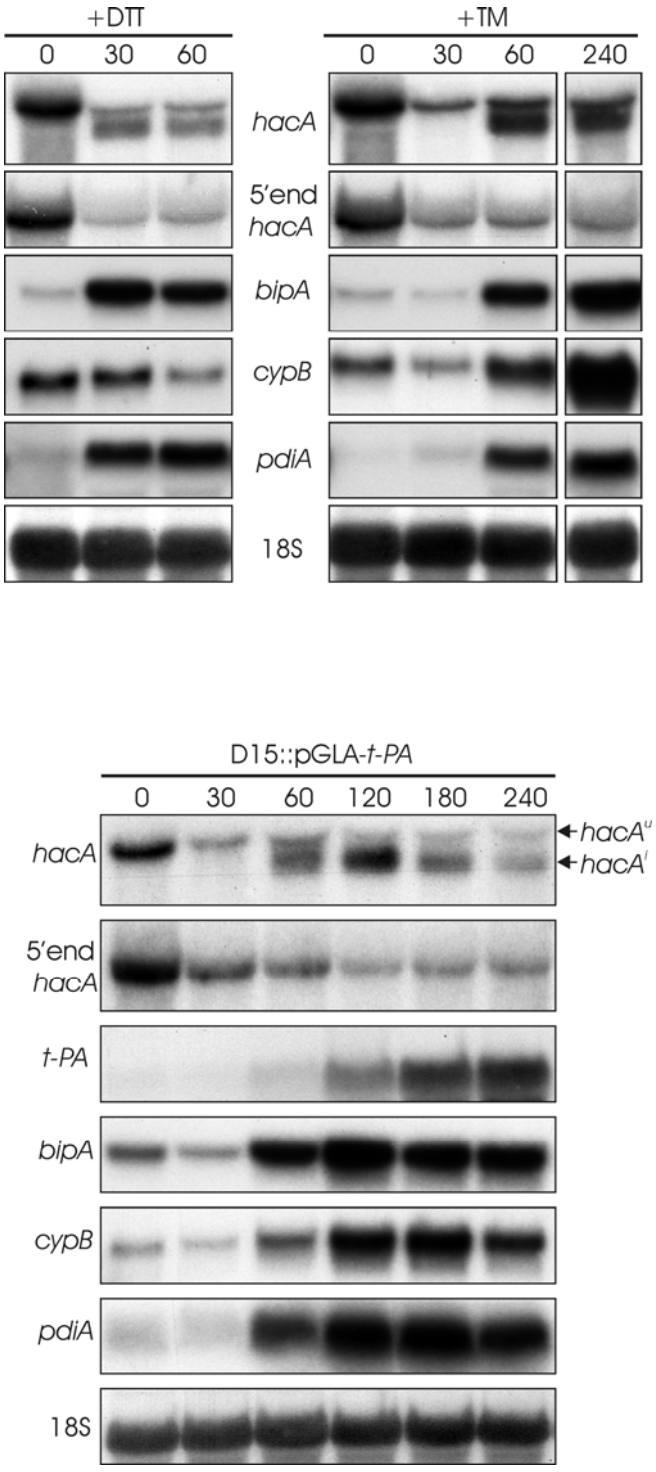


**Figure 5.** Northern hybridization analyses showing the effects of ER stress, imposed by treatment of cells with DTT or tunicamycin (TM) and by the expression of a heterologous gene (D15::*pGLA*-t-PA), on the expression of *hacA*, *bipA*, *cypB*, and *pdiA*. The time in minutes after induction is indicated above the lanes. The two *hacA* mRNA species that were present after induction of ER stress are indicated by the arrows, and are labelled $hacA^u$ and $hacA^i$, for uninduced and induced, respectively. The gene coding for 18S rRNA was used as a control probe.

To examine the UPR induced by the synthesis of t-PA, or by treatment with DTT or tunicamycin, Northern hybridisation was performed on total RNA isolated from mycelia of such cultures. A cDNA fragment comprising the region from the start codon up to the unconventional intron was used as a probe to detect *hacA* mRNA. Northern analysis showed that a second, smaller *hacA*$^{i}$ mRNA transcript emerged within 30 minutes after exposure of the mycelium to DTT, and this coincided with a decrease in the intensity of the *hacA*$^{u}$ band (Fig. 5). Together with the appearance of the smaller *hacA*$^{i}$ mRNA, upregulation of the ER chaperone-encoding gene *bipA* [168], and the foldases-encoding gene *pdiA* [104] was observed. Although some fluctuations in the mRNA levels for the ER foldases CypB [27] were observed, its expression was not obviously affected by the DTT treatment. In contrast, tunicamycin treatment had a profound effect on the expression of *cypB*, and led to a seven-fold upregulation within 4 h (phosphor-imager data, results not shown). Like DTT treatment, tunicamycin treatment also led to the appearance of the *hacA*$^{i}$ band, and the simultaneous upregulation of *bipA* and *pdiA*, although the effect appeared later than in the DTT treated mycelia. Northern analysis of *A. niger* D15::Pgla-t-PA#19 showed that the effect on the UPR was already manifested by in the up-regulation of *bipA*, *cypB* and *pdiA*, and the formation of a smaller *hacA*$^{i}$ band, before clear induction of t-PA could be detected (after 2 h of induction), underscoring the sensitivity of the UPR. Northern blot analysis also revealed that the transcript levels of *bipA*, *cypB* and *pdiA* increased in parallel with the increase in the t-PA transcript. From these results it appears that the effects of ER stress, imposed by the expression of a heterologous protein more closely resembles that caused by tunicamycin than that by treatment with DTT. This is valid for both the response time, and the transcription levels of *bipA*, *cypB* and *pdiA*. The total amount of the *hacA* transcript is lower at $t_{30}$ compared to $t_0$ in all three ER stress treatments. This could be the result of transcriptional down-regulation due to the transfer of mycelia at the start of the experiments. Another possible explanation is that the processed *hacA*$^{i}$ mRNA is less stable than the uninduced *hacA*$^{u}$. This could give the fungus the ability to shut down the UPR more rapidly when the ER stress has been relieved.

**5'-Truncation of *hacA* mRNA**

Splicing of the 20-nt non-conventional intron upon UPR induction was demonstrated by comparative analysis of the cDNA and genomic sequences. However, this splicing event alone cannot account for the difference in size between the *hacA*$^{u}$ and *hacA*$^{i}$ transcripts observed by Northern analysis. Sequence analysis of the clones obtained from the *A. niger* cDNA library indicated that *hacA*$^{i}$ might also be truncated at the 5'-end relative to *hacA*$^{u}$. Furthermore, the *A. nidulans* and *T. reesei hacA/hac1* mRNAs are cleaved within the 5'-flanking region upon UPR induction. However, neither the mechanism nor the exact site of cleavage has been determined in those cases [140]. To confirm that truncation of the 5'-end of

*hacA* mRNA takes place during UPR in *A. niger*, Northern blots were probed with a 230-bp fragment comprising the most upstream part of the *hacA* 5'UTR (Fig. 5). As expected, this probe only hybridises to the full-length $hacA^u$ mRNA, and the signal decreases upon induction of the UPR. Furthermore, this probe does not hybridise to the $hacA^i$ mRNA. This confirms that the $hacA^i$ transcript also lacks a 5'-segment of the $hacA^u$ mRNA. To determine the site of truncation, and to confirm the splicing of the 20-nt unconventional intron from the *A. niger hacA* mRNA under UPR conditions, a 5'RACE experiment was performed. The reactions were performed on RNA extracted from *A. niger* D15::pglaA-t-PA before, and 4 h after UPR induction (Fig. 5). A gene-specific primer, located downstream of the unconventional intron, was used to amplify the *hacA* cDNA. A single band was obtained from a mycelial sample harvested before induction. Two bands were obtained from the sample harvested after induction; the larger of these two bands had the same size as the fragment obtained before induction. This is in agreement with the Northern blot data, which indicated that two *hacA* mRNA species ($hacA^u$ and $hacA^i$) were present 4 h after induction (Fig. 5). All the fragments obtained were cloned and sequenced. The fragment derived from the sample taken before induction was identical to the larger fragment from the sample taken 4 h after t-PA induction ($hacA^u$), and contained the unconventional 20-nt intron. The 5'-ends were located 304 nt upstream of the *hacA* start codon (Fig. 6).

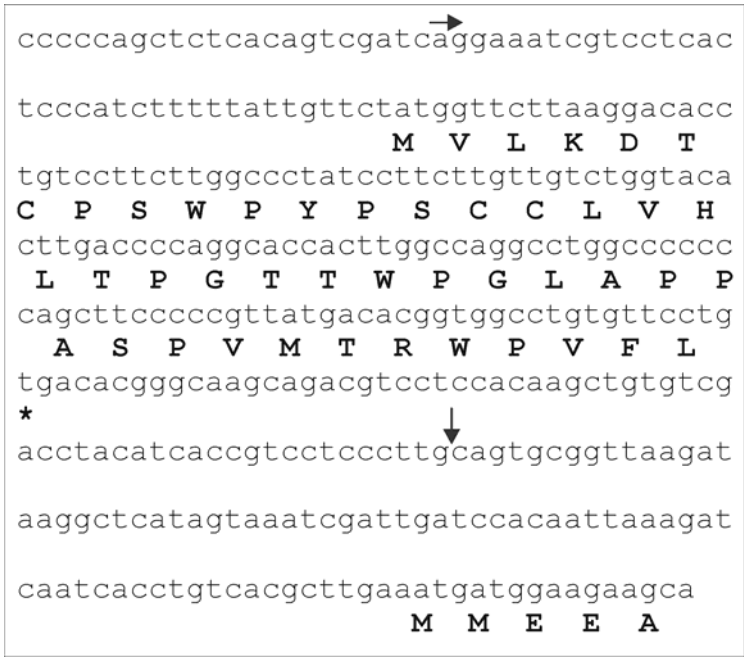


**Figure 6.** Overview of the sequence at the 5'UTR of the *A. niger hacA* gene. The first five amino acids of HacA are indicated in upper case letters. The transcription start site of $hacA^u$ is indicated by the horizontal arrow, and the start site of $hacA^i$ by the vertical arrow. The ORF encoding a 44-amino acid peptide is also indicated in upper case.

Sequence analysis of the smaller fragment, obtained from the induced sample (*hacA*$^{i}$), revealed that its 5'-end lies 230 nt further downstream, only 74 nt upstream of the *hacA* start codon (Fig. 6). Furthermore, the unconventional 20-nt intron was not present in these fragments, confirming that splicing had taken place. The part removed from the 5'UTR of *hacA* contains a short ORF encoding a 44-amino acid peptide. Although it is not known whether this extra region is functional, it could imply the involvement of a translational control mechanism. Interestingly, further inspection of the sequence around the truncation site revealed a sequence stretch similar to those found around the sequence borders of the 20-nt intron (Fig. 3).

**Over-expression of *hacA***

To determine whether over-expression of *hacA*$^{i}$ would mimic the effects of t-PA expression, DTT treatment or tunicamycin treatment, the *hacA* coding region was cloned in an *Aspergillus* expression vector, under the control of the constitutive *A. nidulans gpdA* promoter, and the resulting plasmid (pHM50) was introduced into the genome of *A. niger* N592.

Southern analysis of two transformants detected two additional copies of the *hacA* gene in N592::pHM50#1, and one additional copy in N592::pHM50#2, relative to wild type (Fig. 7). Northern analysis of these strains showed substantial accumulation of *bipA*, *cypB*, and *pdiA* mRNAs in N592::pHM50#1 (Fig. 8a), and a minor up-regulation in the single-copy integrant N592::pHM50#2. These results were confirmed by Western analysis (Fig. 8b), and are analogous to the results obtained upon treatment with tunicamycin and induction of t-PA expression. Quantification of the *hacA*$^{u}$ mRNA by phosphor-imager analysis (data not shown) revealed four- and two-fold higher signals in N592::pHM50#1 and N592::pHM50#2, respectively, compared to wild type. This up-regulation of *hacA*$^{u}$ upon over-expression of *hacA*$^{i}$ suggests that, under UPR conditions, transcription of the *hacA* gene is up-regulated by its own gene product.

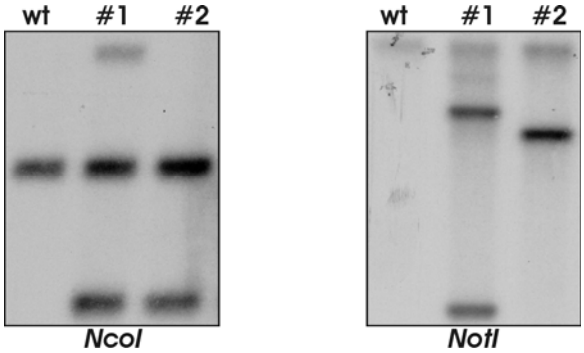


**Figure 7.** Southern analysis showing two additional copies of the *A. niger hacA* gene in strain N592::pHM50#1, and one additional copy in N592::pHM50#2, compared to the wild-type strain N592.

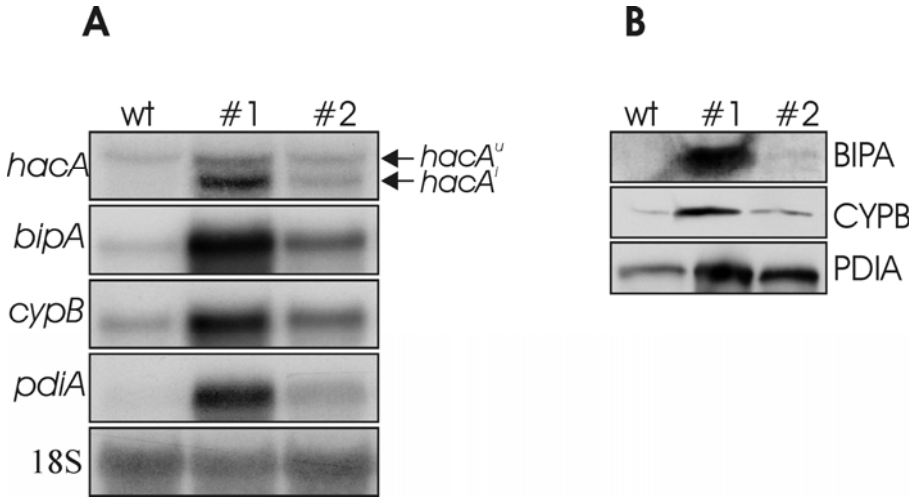


**Figure 8.** Effects of over-expression of *hacA*$^{i}$ on the expression of *bipA*, *cypB*, and *pdiA*. **(A)** Northern analysis showing the effect of over-expression of *hacA*$^{i}$ in *A. niger*. Probes used in hybridisations are indicated on the left. (Lanes: wt, wild-type *A. niger* strain N592; #1, *A. niger* strain N592::pHM50#1; #2, *A. niger* strain N592::pHM50#2). The arrows indicate the two *hacA* mRNA species *hacA*$^{u}$ and *hacA*$^{i}$. Note that the exposure time for the *hacA* blot was reduced relative to that used for Fig. 5, due to the high expression level in strain #2. **(B)** Western analysis showing the effect of *hacA*$^{i}$ over-expression on the levels of BipA, CypB and PdiA protein. Antibodies are indicated in upper case letters on the right.

**Electrophoretic Mobility Shift Analysis (EMSA)**

The experiments described so far show that up-regulation of ER resident chaperones and foldases occurs synchronously with processing of *hacA* mRNA during ER stress. Similar results were obtained without secretion stress upon over-expression of *hacA*$^{i}$, suggesting that HacA is the factor responsible. Whether HacA indeed binds to the promoters of these up-regulated genes was studied *in vitro* by EMSA. Purified HacA protein was used to evaluate binding to the promoters of *bipA*, *cypB*, *pdiA* and *hacA* itself, and to the promoters of two additional ER foldase-encoding genes, *prpA* and *tigA*. *prpA* is homologous to the protein disulfide isomerase gene *pdiA*, and it was shown to be up-regulated during heterologous protein biosynthesis [174]. The product of *tigA* also belongs to the PDI family, and its transcription is induced upon treatment with tunicamycin [62]. The promoter of *cypA*, which encodes a cytoplasmic cyclophylin [26], was used as a negative control. Band-shifts were observed for all the ER-specific promoters (Fig. 9). Interestingly, binding was also observed with a *hacA* promoter fragment. This is in accordance with the finding that over-expression of *hacA*$^{i}$ leads to up-regulation of *hacA*$^{u}$.

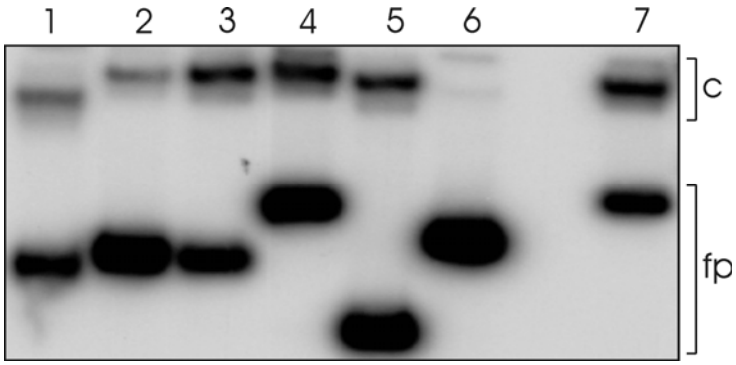


**Figure 9.** Electrophoretic mobility shift analysis (EMSA) showing the binding of the *A. niger* HacA protein to the promoters of genes encoding ER chaperones and foldases, and to the promoter of *hacA* itself (c, protein-DNA complex; fp, free probe). Lanes: 1, P*bipA*; 2 P*cypB*; 3, P*pdiA*; 4, P*prpA*; 5, P*tigA*; 6, P*cypA* (negative control); 7, P*hacA*. The sizes of the promoter fragments used can be deduced from the data in Table 2.

**Cellular Localisation of HacA**

Analysis of HacA using the PSORT II program (http:// psort.nibb.ac.jp/) identified two putative Nuclear Localisation Signals (NLSs), a putative monopartite NLS (PVKKRKS comprising amino acid residues 49– 55), and a putative bipartite NLS (RKRAKTEDEKEQRRIER, amino acid residues 70– 86). The putative bipartite NLS is located in the DNA-binding domain. To examine the cellular localisation of HacA, a hybrid *gfp-hacA* construct was made, consisting of the *hacA*$^{i}$ ORF fused to the 3'-end of the *gfp* ORF. Fluorescence microscopy of N592::pHM52D4, which expresses *gfp-hacA*$^{i}$ under control of the *gpdA* promoter, showed a similar pattern of fluorescence as hyphae stained with the nuclear dye DAPI, confirming that HacA indeed localises to the nucleus (Fig. 10).

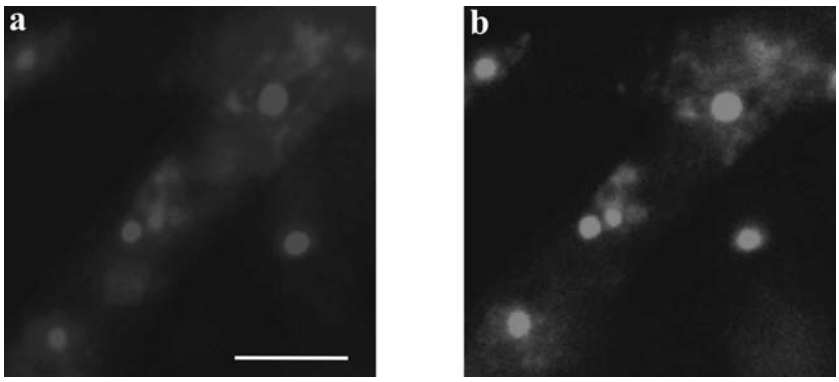


**Figure 10.** Fluorescence microscopy showing the localisation of GFP-HacA. **(A)** Staining of the hyphae with the nuclear stain DAPI. **(B)** Localisation of GFP. The bar represents 10 µm.

# Discussion

The *A. niger hacA* gene was isolated based on its similarity to the *A. nidulans hacA* sequence. The HacA proteins from *A. niger* and *A. nidulans* are very well conserved and are 73% identical. The putative HacA from *A. fumigatus* found in the TIGR genome database (http://www.tigr.org/tdb/e2k1/afu1/) is 80% identical to the *A. niger* HacA. The DNA-binding domains of the three HacA homologues are identical, while their leucine zipper domains are very similar to each other. Sequence differences are mostly located in the N-terminal and C-terminal parts of the proteins.

In yeast, *HAC1* mRNA is expressed relatively abundantly in unstressed cells, but it is not translated due to the presence of a 252-nt intron. This intron pairs with the 5'UTR, thereby preventing the read-through by the ribosomes [137]. Splicing of this intron by Ire1p is necessary for UPR induction [22].

Although the mammalian XBP1 is similar to the yeast Hac1p only insofar as both are bZIP proteins, it has been shown that nearly all features of the yeast splicing pattern are conserved in mammals [186]. However, in contrast to *HAC1*, which is transcribed constitutively, *XBP1* is expressed at low levels and the mRNA is translated, since no inhibitory secondary structure can be formed. Furthermore, activation of the mammalian UPR involves two transcriptional activators, ATF6 and XBP1. Activation starts with the proteolytic release of ATF6 from the ER. ATF6 then up-regulates transcription of genes for ER chaperones, as well as the expression of *XBP1*. *XBP1* is activated by IRE1-mediated splicing of a 26-nt intron, and its protein product up-regulates the same targets as ATF6, including *XBP1* itself. It has been suggested that this system would provide the mammalian cell with a more flexible UPR which is capable of coping more effectively with ER stress [186]. The similarity between the *A. niger* HacA and the yeast Hac1p is confined to the DNA binding domain, and no significant homology could be found between the *Aspergillus* protein and its mammalian orthologue XBP1. Despite these differences, our analysis of the regulation of the *A. niger* HacA indicates that it shares features of both the yeast and the mammalian equivalents, and might even possess features that are specific for the UPR of *A. niger* and other filamentous fungi.

The unconventional introns of *HAC1* mRNA and *XBP1* mRNA are spliced by Ire1p/IRE1 upon induction of the UPR [152,186]. The unconventional 20-nt intron present in the *A. niger hacA*$^{u}$ shows a close resemblance to its counterparts in yeast and mammalian cells (Fig. 3) [12,152]. The sequences at the boundaries of the intron are well conserved, and a similar secondary RNA structure is predicted where the intron borders are encompassed in two loops of 7 nt. This structural and sequence similarity suggests that the *A. niger hacA*$^{u}$ RNA is spliced in a similar manner, most probably by the action of an Ire1p/IRE1 like

protein. We have recently cloned a gene from *A. niger* with similarity to the yeast Ire1p, and preliminary results suggest it is involved in the splicing of *hacA* mRNA.

The only known modification of the yeast *HAC1* and mammalian *XBP1* mRNAs that is triggered by UPR induction is the splicing of the unconventional intron. Results from *A. nidulans* and *T. reesei* [140], and data presented in this paper for *A. niger*, indicate that the *hacA*$^{u}$ mRNA undergoes an additional modification in these fungi. In addition to the unconventional intron-splicing event induced by ER stress, the 5'UTR of *hacA* mRNA is truncated. A 230-nt segment is removed, which contains an ORF with the capacity to code for 44 amino acids. The 5'UTR of the *A. nidulans hacA* gene contains an uORF for seven amino acids, and the *T. reesei hac1* gene contains one uORF for 18 amino acids and one for two amino acids [140]. The presence of these uORFs suggests the involvement of a translational control mechanism. Translational control is often associated with short upstream ORFs in the 5'UTRs of mRNAs [90]. Indeed translation of the yeast transcription factor Gcn4p is controlled by four uORFs [59], and translational control of the synthesis of the transcription factor ATF4, which is involved in the mammalian stress responsive pathway, is dependent on two uORFs [51]. The mechanism responsible for the truncation of *A. niger hacA* mRNA upon ER stress is still unknown. Our results revealed sequence similarity between the truncation site in the 5'-portion of *hacA* mRNA and the borders of the unconventional intron (Fig. 3). However, no RNA secondary structure incorporating the putative truncation site in a stable stem-loop could be predicted for the region around the 5'-truncation site. This makes an involvement of IreA in the truncation unlikely. Furthermore, it has been speculated that, upon induction of the UPR, transcription of *hac1* in *T. reesei* might initiate at a new start site, thus giving rise to the truncated *hac1* mRNA [140]. Our recent identification of a *cis*-acting UPR element in the 5'UTR of the *A. niger hacA* gene supports this hypothesis [100].

*A. niger* HacA is most probably involved in the upregulation of its own gene, as purified HacA protein was able to bind to the *hacA* promoter (Fig. 9) Moreover, over-expression of *hacA*$^{i}$ not only led to up-regulation of *bipA*, *cypB* and *pdiA*, but also led to the accumulation of *hacA*$^{u}$ mRNA (Fig. 8). This is in agreement with the finding that in mammalian cells the transcription of *XBP1* is up-regulated by XBP1 protein upon the onset of the UPR [186].

In conclusion, the several lines of evidence presented here strongly indicate that the *hacA* gene in *A. niger* encodes a transcription factor that regulates the UPR.